

AD_____

Award Number: DAMD17-99-1-9178

TITLE: Functional Analysis of TGFRP, a Putative Angiogenic Factor, in Breast Carcinogenesis

PRINCIPAL INVESTIGATOR: Xiao-Fan Wang, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020717 042

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2001	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 00 - 30 Jun 01)	
4. TITLE AND SUBTITLE Functional Analysis of TGFRP, a Putative Angiogenic Factor, in Breast Carcinogenesis			5. FUNDING NUMBERS DAMD17-99-1-9178	
6. AUTHOR(S) Xiao-Fan Wang, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University Medical Center Durham, North Carolina 27710 E-Mail: wang0011@mc.duke.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words)				
14. SUBJECT TERMS Breast Cancer				15. NUMBER OF PAGES 11
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4-6
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusions.....	6
References.....	N/A
Appendices.....	7

INTRODUCTION

The primary goal of this project is to test the hypothesis that a newly identified protein, TGFRP, functions as an angiogenic factor to promote tumor progression and metastasis. As discussed in the research proposal, our preliminary results strongly support this postulation. With the generation of additional substantial experimental evidence from the proposed studies, we wish to firmly establish a role for TGFRP in the pathological process of breast carcinogenesis and lay the foundation for the development of novel therapeutics for the treatment of breast cancer.

BODY

Task 1: Determine the expression pattern of TGFRP in different stages of primary breast tumors.

As indicated in the last report, we had planned to conduct a systemic examination of TGFRP expression in primary breast tumors in collaboration with Dr. J. Iglehart's group at the Surgery Department of Duke University Medical Center. However, Dr. Iglehart left Duke unexpectedly last July to head the Breast Cancer Research Group at Harvard Medical School and Dana-Faber Cancer Institute. Although we have consequently not been able to pursue this task as originally planned, we have examined the expression pattern of TGFRP during mouse embryonic development. The main goal of doing this is to determine if TGFRP is expressed by any normal tissues or organs. Our preliminary results so far strongly suggest that TGFRP is not expressed in any stage of mouse embryonic development since we have failed to detect any signal in all the tissue sections examined (data not shown). However, we have found that TGFRP is primarily expressed in the bone tissue of adult mice (data not shown). This result suggests that the normal physiological function of TGFRP may be associated with the remodeling process of bone tissue.

Task 2: Determine if TGFRP affects the proliferation, migration and morphogenesis of endothelial cells and induces the formation of blood vessels.

As shown in the section of preliminary results of the original proposal, the tumor phenotype of TGFRP-overexpressing CX-1S cells in nude mice implicate the involvement of enhanced growth of tumor vasculature. This in turn suggests that TGFRP may not act in an autocrine fashion on tumor cells, but in a paracrine manner by affecting endothelial cells. Solid tumor growth and invasion are considered to be exclusively dependent on neovascularization termed angiogenesis. The process of tumor angiogenesis is the response of microvascular endothelial cells to angiogenic molecules such as VEGF and fibroblast growth factor. The angiogenic process including endothelial cell proliferation, extracellular matrix protein degradation, cell migration and vascular tube formation, is initiated by the activation of tyrosine kinase receptors specifically for angiogenic factors on the cell membrane. Flk-1/KDR is one of the major tyrosine kinase receptors which is mainly expressed on endothelial cells and is recognized to conduct VEGF biologic activity for cellular early response including cell mitogenesis and migration. Tie1 and tie2, the other endothelial-associated tyrosine kinase receptors, are also involved in the regulation of angiogenic activity, particularly in blood vessel sprouting, integrate and stabilization.

To investigate if TGFRP has an effect on microvascular endothelial cells, the primary cell type believed to be involved in tumor angiogenesis, we initially utilized a human microvascular endothelial cell line (HMEC-1) as a primary target cell to investigate whether TGFRP might trigger the angiogenic response. HMEC-1 was established through the introduction of an SV-40 large T-antigen for cell immortalization. Although the cell line retains angiogenic function, the morphologic and functional characteristics of HMEC-1 could not precisely represent the primary human microvascular endothelial cells. Our result in Figure 1 shows that HMEC-1 migration was induced by angiogenic factor VEGF and was markedly increased by 2-3 fold following treatment with TGFRP (please note that the term OSF-2 was used in place of TGFRP in all figures). Unexpectedly, TGFRP slightly inhibits HMEC-1 proliferation. In the context of previous study demonstrating that HMEC-1 contains endothelial specific receptor CD31, we also examined other endothelial-associated tyrosine kinase receptors, Flk-1/KDR, tie1 and tie2. Interestingly, we found that Flk-1/KDR was absent in HMEC-1 but present in primary endothelial cells, although both cell lines expressed tie1 and tie2 (Figure 1). More importantly, TGFRP failed to induce Flk-1/KDR, tie1 and tie2 expression in HMEC-1. Those results suggest that physiological function of HMEC-1 may be dissimilar to the primary cells. To overcome the problem of using primary endothelial cells with very limited capability of only a few passages, we immortalized these endothelial cells via introduction of the human telomerase catalytic subunit gene (hTER) into the cells. The immortalized human microvascular endothelial cells (IHMVEC) have been used in our experiments for over 20-30 passages without a loss of cell viability and growth, and they maintain the primary endothelial cell features. For example, IHMVEC retains the angiogenic activity of migration response stimulated by TGFRP (Figure 2). The inhibitory effect of TGFRP on mitogenicity in HMEC-1 is also reproduced in IHMVEC. Moreover, treatment of IHMVEC with TGFRP results in a phenotype of extensive tube formation associated with the development of microvascular structure (Figure 2). These data suggest that TGFRP can induce an angiogenic response in endothelial cells in a similar manner as VEGF, a potent angiogenic factor. In addition, those results demonstrate that IHMVEC represents an excellent in vitro endothelium model system for angiogenic studies.

To further explore the molecular mechanism of TGFRP-induced angiogenesis, we examined whether the regulation of expression of certain known angiogenic factors or their tyrosine kinase receptors play an important role in TGFRP mediated tumor angiogenesis. VEGF and Flk-1/KDR have been demonstrated to induce angiogenesis in the process of tumor progression and invasion. VEGF secreted from tumor cells exerts its biologic effects on endothelial cells via a paracrine loop. We found that the production of VEGF in conditional medium from TGFRP-overexpressed MCF-7 cells was no difference from the medium from control cells (data not shown). Furthermore, TGFRP treatment of MCF-7 cells that lack the expression of endogenous TGFRP did not enhance VEGF production (data not shown). However, incubation of IHMVEC with TGFRP resulted in an up-regulation of Flk-1/KDR, tie1 and tie2 in a dose- and time-dependent manner (Figure 3). In addition, the conditional medium from TGFRP-overexpressed MCF-7 cells induced Flk-1/KDR expression in IHMVEC (Figure 3). To determine if the up-regulation of Flk-1/KDR has a significant impact on TGFRP-mediated angiogenesis, we pretreated IHMVEC with TGFRP and then measured cellular mitogenic and angiogenic responses to VEGF. As shown in Figure 4, cell proliferation induced by VEGF in a

dose-dependent response curve was up-shifted. Consistent with this notion, cell migration induced by VEGF was also potentiated by TGFRP pretreatment (Fig. 4).

As described above, we have so far focused our studies on the effect of TGFRP on endothelial cells in vitro. We are now concentrating our effort on the determination of a protective effect of TGFRP on tumor cells in response to hypoxic conditions.

Task 3: Identify and isolate the receptor/binding target of TGFRP.

Since we have focused our main effort on the determination of biological effects of TGFRP on endothelial cells, as well as tumor cells, we have not made significant progress on the front of receptor identification and isolation. However, we have initiated pilot experiments to examine the feasibility of using Baculo-virus produced recombinant TGFRP as a probe to identify the binding target of TGFRP on cell surface. We have tried to iodinate the recombinant protein and used it in binding assays on HUVEC cells, but the results were not satisfactory due to the high background generated by the non-specific binding of the labeled protein. We are now in the process of generating biotin-labeled TGFRP protein for the same purpose. We are also considering the strategy of generating TGFRP overproduced by mammalian cells through recombinant adenoviral infection. We take this latter approach because of the concern that, even though we have observed specific biological activities from the Baculo viral-produced TGFRP protein, the insect-derived recombinant protein may still be different in certain biological properties from the tumor cell-produced TGFRP. Thus, we would like to make certain that the protein used in the biochemical assay for identifying the binding target will possess the same biological potency of the tumor cell-generated TGFRP protein.

KEY RESEARCH ACCOMPLISHMENTS

1. Preliminary results suggest that TGFRP may act as a novel angiogenic factor for endothelial cells.
2. Preliminary results suggest that TGFRP may promote angiogenesis by upregulation of VEGF receptors in endothelial cells.

REPORTABLE OUTCOMES

All category of activities are in progress.

CONCLUSIONS

The results of our preliminary studies, presented in both the original proposal and this annual report, strongly support the notion that TGFRP functions as a novel factor possessing tumor promoting activities, particularly angiogenic activity, which are intimately associated with the multistep pathological process of tumor angiogenesis, progression and metastasis. To focus our main effort on the tasks of determining the biological effects of TGFRP in angiogenesis, we

would like to modify our future research plan in the next year by putting the original tasks 1 and 3 on lower priorities.

APPENDICES

Four figures which are referenced in the Body text of the report.

HMEC-1	
C	OSF-2

HMEC-1	HMEC-1
1	2

Flk-1/KDR —

Tie 1 —

Tie 2 —

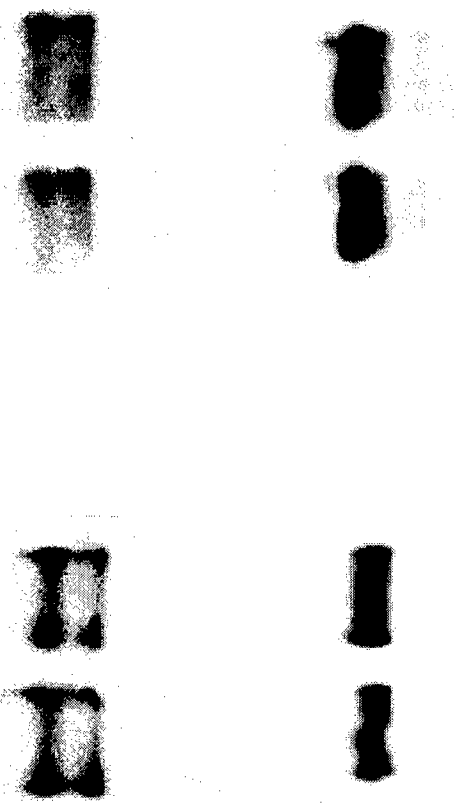
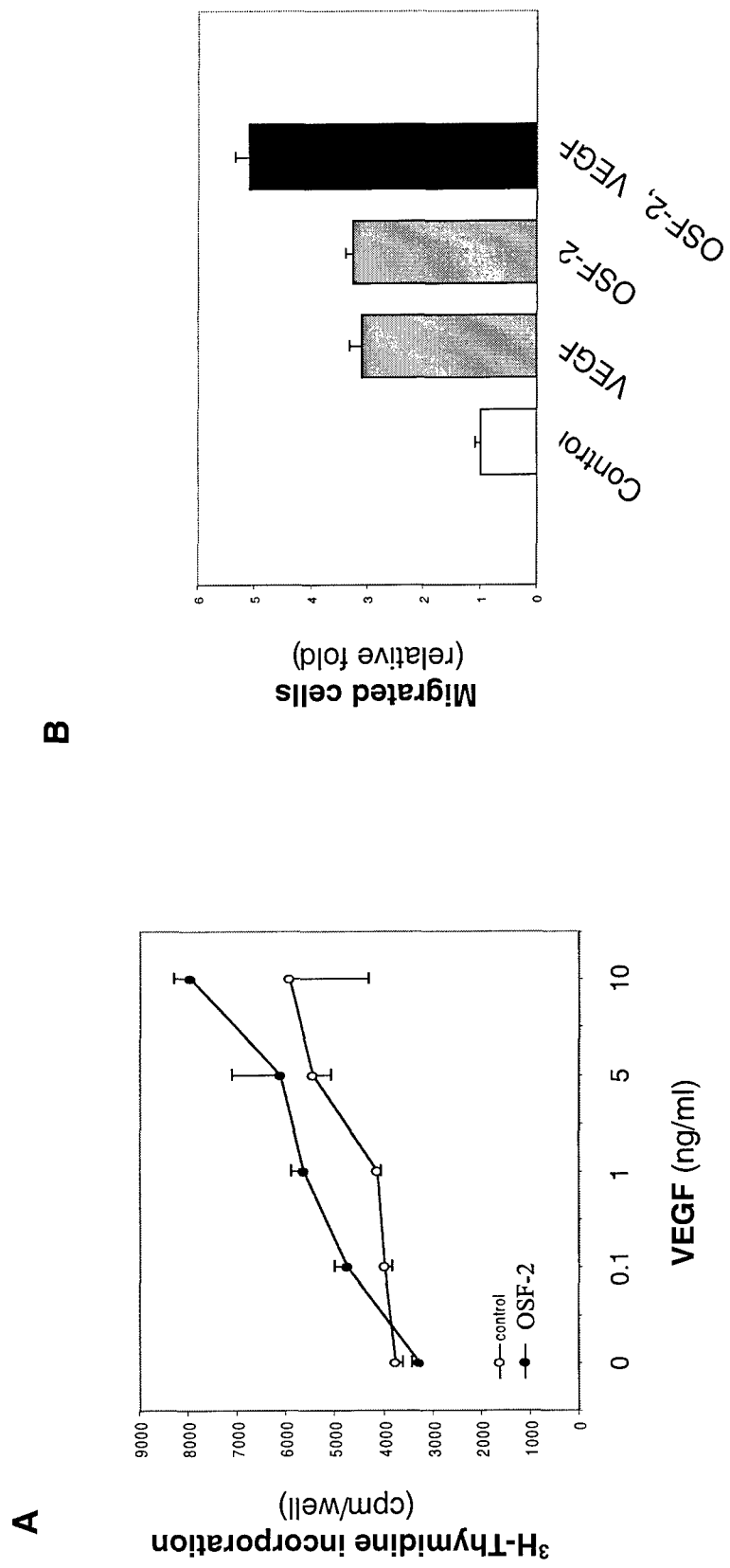
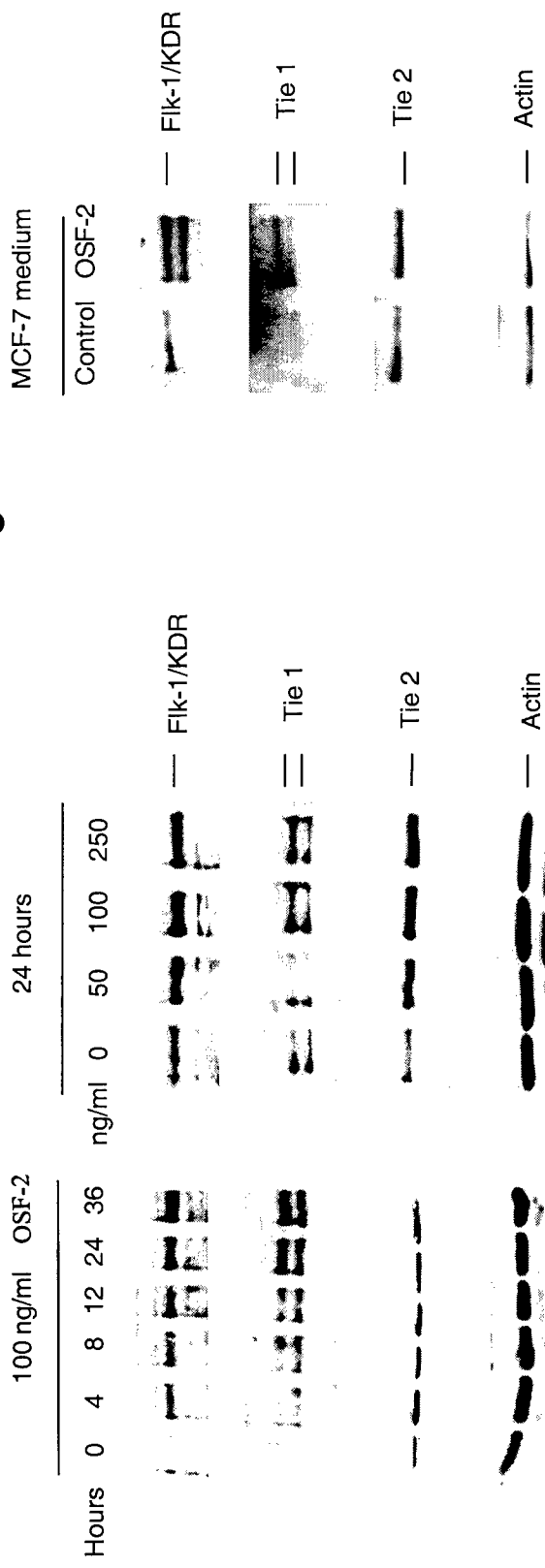


Fig 1

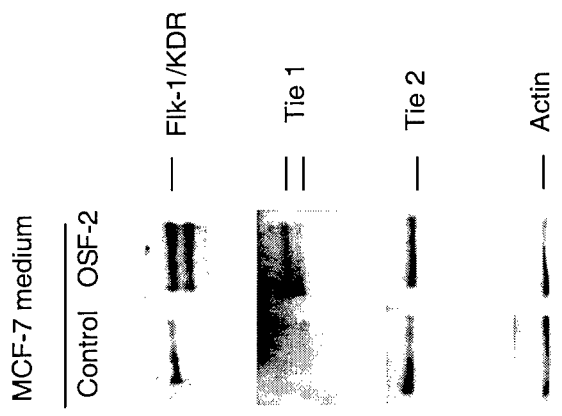
Fig 2



A



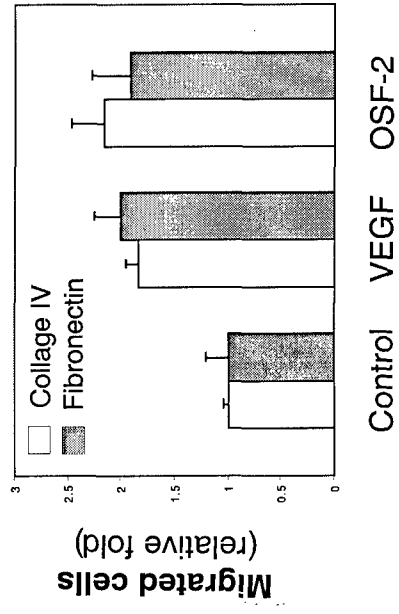
C



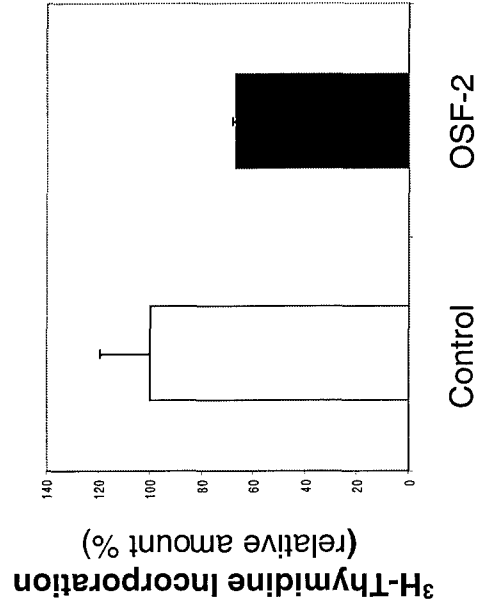
B

Fig 3

A



B



C

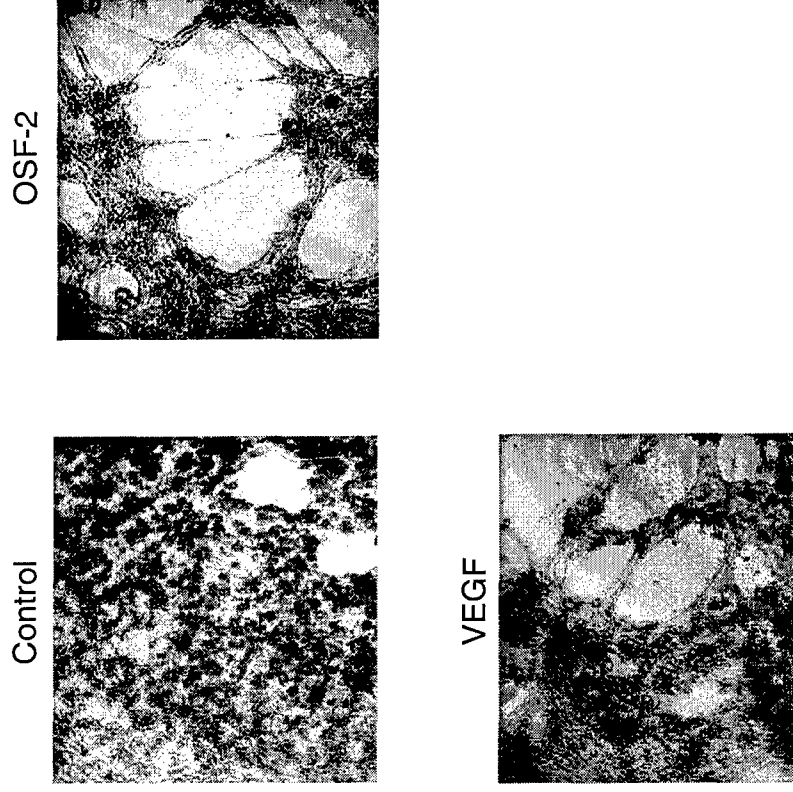


Fig 4